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Studies on the Fate of Defined Asialoglycoproteins in the Circulation of the Mouse

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Summary: Asialoglycoproteins injected into the tail vein of mice accumulated rapidly in the liver, as found in rats and rabbits by other authors, whereas the native glycoprotein remained in the serum for much longer periods of time.

Histological examination of liver sections after the injection of radiolabelled asialoglycoproteins failed to show any binding to the hepatocyte membrane. However, silver grains were observed in the sinuses of the liver and often appeared to be bound to the sinus wall. In addition, small amounts of label in the reticulo-endothelial system of the spleen were observed.

A comparison in the uptake of asialoglycoproteins by the liver, between those with alkali-labile galactose-containing (AL) chains and those with alkali-stable galactose-containing (AS) chains, showed that the former were taken up more slowly. There was no correlation between total galactose content and rate of hepatic uptake.

Asialoglycoproteins with AS chains could not inhibit the uptake in the liver of those with AL chains. However, when the order was reversed, the uptake of AS containing asialoglycoproteins was inhibited by 50 %. Different mechanisms to explain these results are discussed.

Untersuchungen über das Schicksal definierter Asialoglykoproteine im Kreislauf der Maus

Zusammenfassung: Asialoglykoproteine, welche in die Schwanzvene von Mäusen injiziert wurden, akkumulierten sehr schnell in der Leber, was auch von anderen Autoren bei Ratten und Kaninchen gefunden worden ist, wohingegen das native Glykoprotein für einen viel längeren Zeitraum im Serum verweilte.

Histologische Untersuchungen an Leberschnitten nach der Injektion von radioaktiv markierten Asialoglykoproteinen zeigten allerdings keine Bindung an der Hepatocytenmembran, jedoch konnten Silberkörnchen in den Lebersinus beobachtet werden, die oft an den Sinuswall gebunden zu sein schienen. Außerdem wurden kleine Mengen an markierter Substanz in dem retikulo-endothelialen System der Milz festgestellt.

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Ein Vergleich hinsichtlich der Aufnahme von Asialoglykoproteinen durch die Leber wurde in Bezug auf solche mit alkali-labilen, galaktosehaltigen (AL) Ketten und solche mit alkalistabilen galaktosehaltigen (AS) Ketten durchgeführt, wobei sich herausstellte, daß die ersteren viel langsamer aufgenommen wurden. Es ergab sich aber keine Korrelation zwischen dem Gesamt-Galaktosegehalt und der Rate der Aufnahme durch die Leber.

Asialoglykoproteine mit AS-Ketten konnten die Aufnahme in die Leber von solchen mit AL-Ketten nicht inhibieren. Jedoch in umgekehrter Reihenfolge konnte die Aufnahme von Asialoglykoproteinen mit AS Ketten zu 50 % gehemmt werden. Diesen Ergebnissen liegen wahrscheinlich verschiedene Mechanismen zugrunde.

Introduction

Removal of sialic acid from many glycoproteins followed by intravenous injection results in a rapid uptake by the liver and subsequent removal from the serum (1,2). This is in contradistinction to the native glycoprotein which remains in the serum for long periods of time. The mechanism whereby asialoglycoproteins are sequestered in the liver has been shown to be dependent, in mammals, on the integrity of terminal *D*-galactosyl residues, which are usually exposed after removal of terminal sialic acid (1). In birds there appears to be a comparable system involving terminal N-acetyl-*D*-glucosamine (3).

In addition to a galactose-specific recognition system in mammalian liver, there is some evidence that rats may possess receptors for *D*-mannose and N-acetyl-*D*-glucosamine on the *Kupffer* cell surface in the liver (4). *L*-Fucose will also compete with these two latter monosaccharides for uptake by the liver (4), suggesting that either there are several recognition systems involving different sugars or that a single receptor exists which is unable to distinguish between various glycosyl configurations. Galactose, however, does not affect the uptake of mannose or N-acetyl-glucosamine suggesting a distinct receptor for this monosaccharide, although it has been shown that the *D*-galactose receptor cannot distinguish between the galactosyl and glucosyl configurations (5).

Asialoglycoproteins usually possess terminal *D*-galactosyl residues, which in turn are linked usually to N-acetyl-glucosamine or N-acetyl-galactosamine via a $\beta 1-4$ or $\beta 1-3$ linkage respectively (6). These two types of linkages are often indicative of the carbohydrate chain involved. For example, carbohydrate chains of the so-called serum type are usually branched, linked to protein via an N-acetyl-glucosamine/asparagine bond, contain mannose and often have the terminal sequence NANA-Gal($\beta 1-4$)-GlcNAc[2]) (7). In contrast the smaller mucin type of chain has an alkali-labile N-acetyl-galactosamine/serine or threonine linkage to the protein backbone, does not contain mannose and often has the terminal sequence NANA-Gal($\beta 1-3$)-GalNAc (GalNAc can also be substituted by NANA) (8). Investigations into the fate of asialo serum glycoproteins, which contain both of these types of carbohydrate chain, may be affected therefore not only by the presence of terminal *D*-galactosyl residues but also by the influence of the sub-terminal sugar and its linkage.

The fate of asialoglycoproteins with the terminal sequence Gal($\beta 1-3$)GalNAc. . . is especially interesting as it has been shown that there exists, in most mammals, a naturally occurring antibody directed against this structure (9,10). This study was undertaken therefore to examine the clearance of asialoglycoproteins in which the terminal galactosyl residues are present as part of a serum type (alkali-stable) or the mucin (alkali-labile) type of carbohydrate chain and also whether separate mechanisms exist for their removal from the serum.

[2]) Abbreviations

GalNAc:	N-Acetyl-D-galactosamine
GlcNAc:	N-Acetyl-D-glucosamine
NANA:	N-Acetyl neuraminic acid
Gal(1–3)GalNAc:	D-Galactosyl(1–3)-N-acetyl-D-galactosamine.
AS:	Alkali-stable (carbohydrate chain)
AL:	Alkali-labile (carbohydrate chain)

Experimental

Materials

An inbred strain of MRI mice originally purchased from the Süddeutsche Versuchstier-Farm, Tuttlingen were used in all experiments. Human coeruloplasmin, cholinesterase, orosomucoid (α_1 acid-glycoprotein), haptoglobin, β_2 III glycoprotein, lactoferrin as well as *Vibrio cholerae* neuraminidase (E.C. 3.2.1.18, 500 units/ml) and all general chemicals were obtained from Behringwerke (Marburg/Lahn, F.R.G.).

Antifreeze glycoproteins from the Antarctic fish *Trematomas borchgrevinki* with molecular weights of between 10,500 and 23,500 were isolated as described previously by *De Vries* et al. (11).

Galactose oxidase (E.C. 1.1.3.9) was obtained from Sigma Ltd., and tritiated acetic anhydride from NEN Chemicals (specific activity 370 GBq/mmol). Sodium borotritiide (specific activity 740 GBq/mmol) was obtained from Amersham/Searle.

Methods

Desialylation of glycoproteins

Glycoproteins (4.5 mg) were dissolved in phosphate buffered saline (1 ml) and incubated for 24 h at 37 °C with 40 units of neuraminidase in the presence of 10 mmol/l sodium azide at pH 5.6. Preliminary experiments with all the glycoproteins used showed that these conditions were sufficient to release all the sialic acid. Antifreeze glycoprotein was the only exception as it did not contain any sialic acid.

Alternatively, glycoproteins were incubated for 1 h at 80 °C in the presence of 0.05 mol/l H_2SO_4, after which the solution was

cooled and neutralised with Dowex-1 resin ($-OH^-$ form). The resin was removed by centrifugation and the supernatant either stored at −20 °C or lyophilised then stored. Although identical results were obtained with either method, treatment with low concentrations of neuraminidase was preferred.

Monosaccharide analysis of glycoproteins

Monosaccharide analysis was carried out as previously described (12). Briefly, glycoproteins (1 mg/ml) were incubated with 3 mol/l HCl at 100 °C for 4 h, after which the solution was neutralised with silver carbonate. The precipitate of silver chloride was removed by centrifugation. The supernatant and washings were lyophilised, reacetylated (13) and trimethylsilyl derivatives prepared for chromatography (12). An internal standard of *D*-erythritol (30 µg) was included before hydrolysis and the trimethylsilyl-monosaccharides measured by gas chromatography on columns of 3 % OV-17 and 3 % SE-30 on Chromasorb Q (80–100 mesh, Serva).

Sialic acid was not routinely determined by gas chromatography but by the method of *Aminoff* after hydrolytic release in 0.05 mol/l H_2SO_4 at 80 °C for 1 h (14).

Removal and measurement of the alkaline-labile disaccharide β-D-galactosyl(1–3)-N-acetyl-D-galactosamine

Glycoproteins (1 mg) were incubated at 37 °C for 24 h with 0.05 mol/l NaOH containing 1.0 mol/l $NaBH_4$ and then neutralised with Dowex 50 resin ($-H^+$-form). The supernatant was removed and the resin washed with water. Supernatant and washings were combined, lyophilised and trimethylsilyl derivatives prepared for gas chromatography in the same way as for monosaccharides. Before alkaline borohydride treatment, glycoproteins were either desialylated by acid hydrolysis or left untreated in order to determine the amount of disaccharide unsubstituted by sialic acid as well as the total amounts. An internal standard of trehalose was included (30 µl).

Radiolabelling of glycoproteins

Two methods were used for the radiolabelling of glycoproteins; the galactose oxidase/NaB^3H_4 technique, in which carbohydrate residues could be labelled and direct acetylation of the protein chain. For the former method, glycoprotein samples were desialylated as described and then incubated for 20 h at 25 °C with 10 units of galactose oxidase. After oxidation, glycoproteins were reduced by addition of 37–74 MBq of NaB^3H_4 and incubation at room temperature for 30 min. Reduction was completed by adding excess non-tritiated $NaBH_4$ (10 mg) with further incubation for 15 min. The glycoproteins were dialysed against phosphate buffered saline (3 × 5 l), concentrated and volumes adjusted. If not used immediately the aliquots were stored at −20 °C.

The second method of radiolabelling was acetylation with tritiated acetic anhydride as has been previously described (15).

Injection of mice

Varying concentrations of radiolabelled glycoprotein were injected intravenously into mice via the tail vein. After injection the mice were killed by anaesthetising with ether followed by exsanguination and cervical dislocation. This was carried out at periods between 1 and 30 min after injection. Liver, kidney, spleen, blood and urine were routinely removed but in addition from some mice brain, skeletal muscle, heart, lung and bone marrow were also examined. Each organ was weighed immediately after removal, a portion fixed for histological staining and approximately 100 mg taken for radioactive counting.

Measurement of radioactivity in tissues

Organs which were to be counted were cut into small pieces in a glass counting vial and 1 ml of 'NCS Tissue Solubiliser' (Amersham/Searle, England) added. The samples were then left at 40 °C overnight or until the tissue was completely digested. Any discoloration was removed by briefly heating in the presence of a drop of hydrogen peroxide. Scintillant was added and the samples counted. Radioactivity was expressed as counts/min · g of fresh tissue or as counts/min per whole organ.

Inhibition of glycoprotein uptake

Inhibition experiments were carried out to examine the effect of asialoglycoproteins containing the disaccharide β-Gal(1–3) GalNAc on the uptake of asialoglycoproteins that do not contain this disaccharide and vice versa. This was done by injection of a non-labelled glycoprotein followed by a second labelled asialoglycoprotein. The second glycoprotein was injected at the time when the first glycoprotein had reached maximum uptake in the liver, usually 10–15 min. The organs were then excised 10–15 min after the injection of the second glycoprotein and counted as described above.

Histology

Tissue (ca. 100 mg) removed as described above was fixed in 60 g/l formaldehyde and, after embedding in paraffin wax, sectioned, stained by conventional histochemical staining techniques and then prepared for autoradiography. This was carried out by overlaying the preparation with K_2 Emulsion (Ilford) and exposing the samples for 8–15 days before development.

Results

Analysis of glycoprotein mono and disaccharide content

The amounts of monosaccharides present in the various glycoproteins together with the amount of alkali-labile disaccharide β-*D*-galactosyl-1–3)-N-acetyl-*D*-galactosamine is summarised in table 1.

It can be seen that cholinesterase and β_2III glycoprotein both contain the above disaccharide and also are the only glycoproteins to possess N-acetyl-galactosamine residues. In addition they contain some mannose and N-acetyl-glucosamine suggesting the presence of both alkali-labile (AL) and stable chains (AS). Lactoferrin, haptoglobin, orosomucoid and ceruloplasmin contained relatively higher amounts of mannose and N-acetyl-glucosamine, in relation to the total carbohydrate, and no alkali-labile material suggesting that the carbohydrate is present in a serum-type (AS) oligosaccharide chain. Antifreeze glycoprotein was not subjected to analysis because of the small amounts available but it is known from previous data that only galactose and N-acetylgalactosamine are present in a 1:1 ratio and this is linked at every third amino acid in the peptide chain as β-*D*-galactosyl(1–3)-N-acetyl-*D*-galactosamine (16,17,18).

The fate of injected asialoglycoproteins

Removal of 100 % of sialic acid prior to injection resulted in a rapid removal of asialoglycoproteins from the serum, which were then taken up by the liver. Maximum label in the liver was found after 10–15 min after which it decreased to less than 50% of maximum approximately 30 min later. The gradual decrease in the amount of radioactivity in the liver could not be detected as an increase in serum levels, although the amount found in the kidney and especially the urine steadily increased. The radioactivity in the urine was difficult to quantify as the volume in the bladder was different with each mouse.

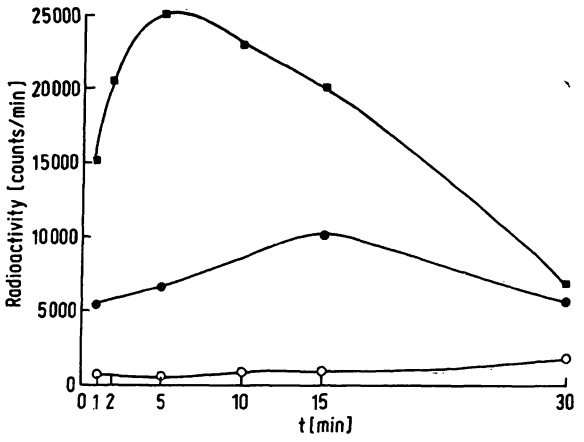

Fig. 1. Uptake of ^3H by the liver at various times after injection of asialo and native ^3H-α_1-acid glycoprotein.
o—o Native α_1 acid glycoprotein; ■—■ Asialo α_1 acid glycoprotein; ●—● α_1 acid glycoprotein minus 25% of the total sialic acid.

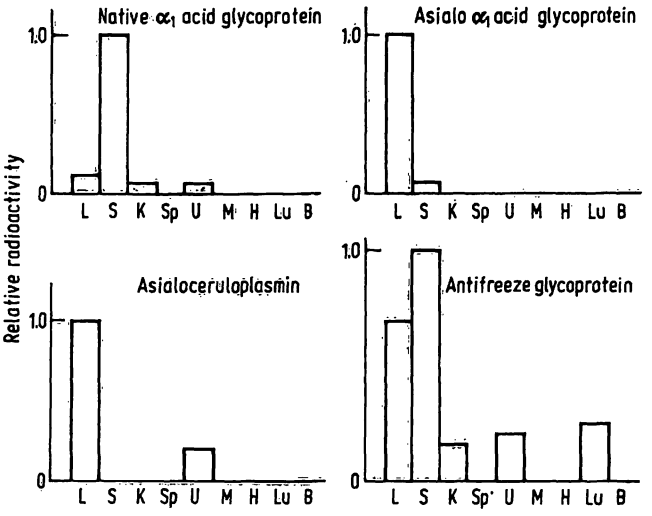

Fig. 2. Distribution of ^3H in various tissues 10 minutes after injection of native and asialo glycoproteins.
For asialo glycoproteins the liver value is fixed at 1.00.
For native glycoglycoproteins serum levels are fixed at 1.00.
L = liver; S = serum; K = kidney; Sp = spleen; U = urine; M = muscle; H = heart; Lu = lung; B = bone marrow.

Removal of only 25 % of the sialic acid also resulted in uptake by the liver although the peak of maximum uptake occurred about 10 min later (fig. 1). When native glycoproteins were injected no uptake was seen in the liver and serum levels fell only slightly over a 30 min period.

No difference in distribution or uptake were found whether glycoproteins were labelled by galactose oxidase/$NaBH_4$ or by acetylation. This indicated that the carbohydrate parts of the glycoprotein molecule were not cleaved in vivo and not the explanation for the decrease in radioactivity in the liver after the maximum peak. As a higher specific activity could be obtained with the direct acetylation technique, this was therefore used for most experiments. Antifreeze glycoprotein was always labelled by the galactose oxidase technique, however.

Tissue distribution of injected glycoproteins

With the exception of antifreeze glycoprotein, counting of tissue digests from various organs showed that after injection of asialoglycoproteins most of the radioactivity was localised in the liver. The kidney (and urine) was the only other organ to accumulate any significant activity. The number of counts in the kidney was low in comparison to the liver, although this increased when higher amounts of glycoproteins were injected. Antifreeze glycoprotein and β_2 III glycoprotein gave higher recoveries in the kidney than the other glycoproteins, however. This was probably due to their lower molecular weights (fig. 2).

After injection of antifreeze glycoprotein some activity was measurable in the lung. This was not observed with any other glycoprotein.

Histological findings

Liver, spleen, bone marrow and heart tissue from mice which had been injected with asialoglycoproteins were examined by autoradiography. Only liver sections showed distinct localised areas of labelling although small amounts of label were present on cells of the reticuloendothelial system in the spleen. Although labelling was detected in the spleen using autoradiography, it was not measurable when pieces of spleen were digested and counted directly in a scintillation counter.

Silver grains in liver preparations were found mainly in the sinus (fig. 3). No direct evidence for the binding of asialoglycoproteins to *Kupffer* cells was obtained, however, areas associated with these cells showed the presence of radiolabelling. Throughout the experimental time course of 30 min, no direct binding to hepatocytes was observed.

Labelling was only seen on the sinusoidal side of the sinus wall. When antifreeze glycoprotein was injected, traces of silver grains were sometimes observed on the surfaces of erythrocytes within the hepatic capillaries.

Effect of chain type on uptake of glycoproteins

From table 1 it can be seen that lactoferrin, haptoglobin, orosomucoid and ceruloplasmin contain no alkali labile disaccharide whereas cholinesterase and β_2 III glycoprotein probably contain both types of chain. Antifreeze glycoprotein, however, contains only the alkaline labile disaccharide. Injection of the first four glycoproteins all gave kinetics of uptake similar to that shown in figure 1. Antifreeze glycoprotein was taken up by the

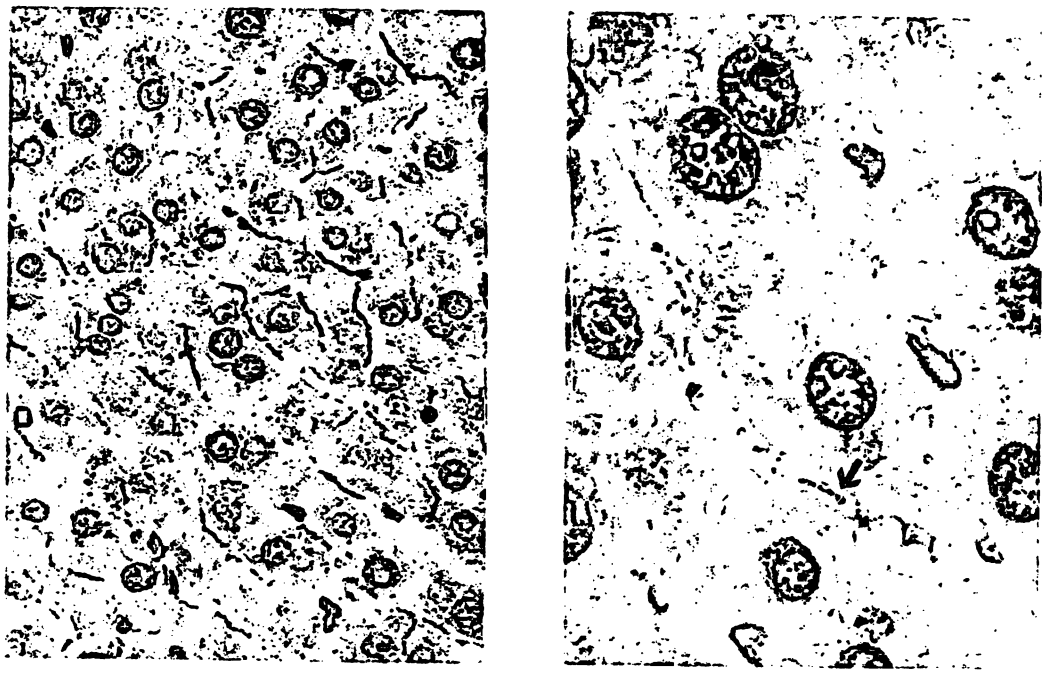

Fig. 3a/b. Autoradiograph of a liver section 10 minutes after injection of ^{3}H-antifreeze glycoproteins. Silver grains can be observed in the sinuses (arrow).

Tab. 1. Monosaccharide and disaccharide analysis of glycoproteins used in uptake experiments

Glycoprotein	Monosaccharide content (mg/g protein)							
	Fuc	Man	Gal	GalNAc	GluNAc	NANA	Total	Gal(1–3) GalNAc
Lactoferrin	7.3	16.1	11.5	0	39.3	7.5	76.3	0
Haptoglobin	2.5	45.1	20.6	0	124.0	44.8	237.0	0
α_1 acid glycoprotein	6.0	53.2	26.2	0	174.0	108.4	367.5	0
Ceruloplasmin	2.5	24.5	13.5	0	66.7	19.7	126.8	0
Cholinesterase	3.0	37.5	21.3	40.3	112.0	64.0	278.1	42.8
β_2III serum glycoprotein	1.0	6.8	6.7	12.0	17.0	16.7	60.1	2.0

liver more slowly. The peak of maximum uptake was consistently observed to be about 5 min later than that of the first four glycoproteins. This did not appear to be related to either the concentration of glycoprotein used or to the amount of galactose in the glycoprotein. Both cholinesterase and β_2 III glycoprotein fell between these two extremes (fig. 4).

Inhibition of the uptake of lactoferrin by antifreeze glycoprotein

Injection of asialo-orosomucoid followed by antifreeze glycoprotein did not affect the uptake of the latter. Similarly prior injection of asialolactoferrin also had no effect on the uptake of antifreeze glycoprotein. By reversing the order of administration it was found that antifreeze glycoprotein could inhibit the uptake of asialolactoferrin by approximately 50 % (tab. 2).

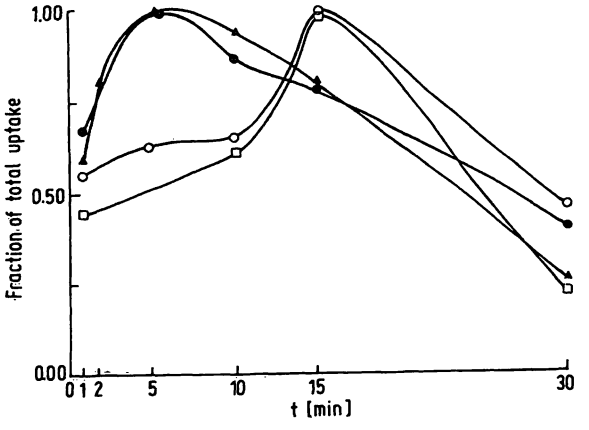

Fig. 4. Uptake of ^{3}H by the liver after injection of asialo glycoproteins with different carbohydrate chains. Uptake is expressed as a percentage of the maximum uptake.
●—● Asialo lactoferrin; ▲—▲ Asialo α_1 acid glycoprotein; ○—○ Asialo β_2III serum glycoprotein; □—□ Antifreeze glycoprotein.

Tab. 2. Inhibition of uptake of ^{3}H-labelled asialo glycoproteins by the liver by non-labelled asialo glycoproteins with different carbohydrate chain type

		Counts/min · g fresh tissue or** Counts/min · ml serum		
1st Glycoprotein	2nd Glycoprotein	Liver	Serum	Kidney
10 µg antifreeze glycoprotein*	–	173	380	564
100 µg antifreeze glycoprotein*	–	1 127	1070	488
100 µg asialo α_1 acid glycoprotein	10 µg antifreeze glycoprotein*	141	490	773
100 µg asialo-lactoferrin	100 µg antifreeze glycoprotein*	1 386	900	915
10 µg asialo-lactoferrin*	–	53 876	12 190	6 175
100 µg asialo-lactoferrin*	–	351 037	94 300	29 729
100 µg antifreeze glycoprotein	10 µg asialo-lactoferrin*	27 826	20 980	9 453
100 µg antifreeze glycoprotein	100 µg asialo-lactoferrin*	262 773	162 260	43 328

* indicates radiolabelled glycoprotein.
** Mean values from 5 animals; for further details see Ute Fricke, Thesis, Medical Faculty, University of Cologne, 1979.

Discussion

The aim of this work was to investigate the fate of asialoglycoproteins with different types of galactose-containing carbohydrate chains, after intravenous injection into mice. The majority of the labelled material was removed very rapidly from the serum and concentrated in the liver in a manner similar to that demonstrated in rats by *Gregoriades* et al. (19). Removal of only a part of the total sialic acid resulted in a slower rate of uptake by the liver, reaching a maximum approximately 5 minutes later after 25 % of the sialic acid had been removed, for example.

The kinetics of uptake of asialoglycoproteins by murine liver were similar to those in the rat, as reported by other authors (19,20). After approximately 30 minutes the levels had returned almost to basal level, suggesting that it was being re-released into the serum or metabolised. No significant radioactivity was found in the serum after this time, however, and the increased amounts in kidney and urine would support the latter hypothesis.

Apart from that found in the kidney (and urine) the majority of radioactivity was found in the liver, when measured by scintillation counting of tissue digests. β_2 III serum glycoprotein and antifreeze glycoprotein appeared in the kidney and urine in higher amounts than any of the other glycoproteins tested, which may be a reflection of the lower molecular weights of these glycoproteins, enabling them to pass directly through the glomerular membrane without prior binding and metabolism in the liver. When higher amounts of glycoproteins were injected, the distribution was identical except that some of the antifreeze glycoprotein localised in the lung. Whether this was due to the presence of a receptor in the lung or whether it is the result of binding of immune complexes to elements of the reticulo-endothelial system (RES) in the lung, is not known.

Examination of autoradiographs of liver sections removed at varying intervals after the injection of asialoglycoproteins suggested that, over a period of 30 minutes, there was no evidence that binding to hepatocytes had occurred. This is in contrast to the findings of *Morell* et al. (1) who reported exclusive binding to the hepatocyte. Labelling was only seen on the sinusoidal side of the sinus wall and at no time had crossed this wall. Binding to the other major cell in the liver, the *Kupffer* cell, was not directly shown, although localised concentrations of label on the sinus wall around the site of these cells was often observed. *Kolb & Kolb-Bachofen* (21) have suggested that soluble asialoglycoproteins as well as asialo cells may be taken up by *Kupffer* cells. The evidence of small amounts of label in the RES of the spleen would seem to support this suggestion. *Van Rijk & van den Hamer* (20) have also reported small accumulations of asialo α_1 acid glycoprotein in rat spleen after 20 minutes.

The elegant series of experiments of *Morell, Ashwell* and coworkers (1,2,22,23,24) have clearly shown that a hepatic receptor can be isolated from rats and rabbits which binds asialoglycoproteins selectively and with high affinity *in vitro*. The results of our experiments, however, have failed to show that asialoglycoproteins, when injected into mice and examined histologically, come into contact with the hepatocyte or indeed cross the sinus wall. However, our biochemical measurements support the existence of a receptor for asialoglycoproteins in the liver.

A comparison of glycoproteins with AL galactose containing chains with those containing AS-galactose chains did not in general show any differences in distribution, with the exception of the finding described above in the lung after high levels of antifreeze glycoprotein. Glycoproteins with AS carbohydrate chains were consistently found to be taken up by the liver more rapidly than those with AL carbohydrate chains. This, however, was not dependant on the total amount of galactose present. Greater numbers of residues per glycoprotein molecule did not necessarily mean a faster rate of uptake.

The possibility of more than one type of receptor specific for galactose has previously been postulated (21) and, although *Ashwell & Morell* examined the competitive inhibition between asialo-orosomucoid and asialo-ceruloplasmin, the existence of different receptors for glycoproteins with different carbohydrate chains was not considered. Our results, using glycoproteins with AL chains to compete with those possessing AS chains suggested that this could be the case although the results can also be explained by other mechanisms. The four main possibilities are as follows:

1. A single receptor exists and that a terminal galactose residue linked β1–3 to GalNAc is in a better conformation to bind than one linked via a β1–4 linkage to GlcNAc.
2. The galactose receptor may have an extended site i.e. one of which galactose is only part and where a second sugar also plays a role. GalNAc may fulfil this function better than GlcNAc.
3. Two receptors may be present both of which bind galactose in the β1–3 linked conformation but only one of which binds galactose residues in the β1–4 linked conformation. Thus prior injection of antifreeze glycoprotein (which has a β1–3 linkage) would block both receptors whereas prior injection of asialolactoferrin or asialo-orosomucoid would only block one, not therefore affecting the binding of antifreeze glycoprotein.
4. The in vivo role of anti-TF can be considered. It is known that antifreeze glycoprotein will bind anti-TF (17) although asialolactoferrin or asialo-ceruloplasmin will not (unpublished results). It is possible therefore that glycoproteins containing the disaccharide Gal (β1–3)GalNAc will form immune complexes in vivo which are then bound by cells in the liver (or other cells of the RES). Thus, antifreeze may bind partly to liver cells via terminal galactose residues and partly as immune complexes. Asialo-orosomucoid and asialolactoferrin will only bind via terminal galactose residues however. Prior injection of asialo-lactoferrin would therefore not inhibit the binding of immune complexes but antifreeze may compete for the latter's binding site as well as forming an immune complex.

The true nature of the binding of different glycoproteins is not possible without further investigation but we have endeavoured to show that the assumption that asialoglycoproteins bind to cells in the liver solely by virtue of their exposed galactose residues may not be the only explanation. Consideration of the type of carbohydrate chain and careful histological examination is a necessary requirement for the investigation of the fate of asialoglycoproteins in vivo.

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